

# Demonstration of three proteins induced by vitamin K absence (PIVKAs) in the cow

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## DEMONSTRATION OF THREE PROTEINS INDUCED BY VITAMIN K ABSENCE (PIVKAs) IN THE COW

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### SUMMARY

(1) With the aid of specific antisera against each of the coagulation factors II (prothrombin), IX and X, it could be demonstrated that three different proteins occur in the plasma of the cow receiving phenprocoumon that are absent in normal plasma. These proteins are called 'proteins induced by vitamin K absence' (PIVKAs).

(2) Each of the three PIVKAs has antigenic determinants in common with only one of the three factors mentioned. We therefore speak of PIVKA-II, PIVKA-IX and PIVKA-X.

(3) In the absence of calcium ions the normal factors and the PIVKAs have the same electrophoretic mobility. In the presence of calcium ions the electrophoretic mobility of the normal factors decreases but that of the PIVKAs remains unaltered.

(4) Quantitative immunoprecipitation and one-dimensional Laurell electrophoresis indicate that the sum of the concentration of normal factors and PIVKA in the plasma of cows receiving phenprocoumon is roughly equal to the concentration of the normal factor in the untreated cow.

(5) PIVKA-II can be converted into thrombin by prothrombinase, although much more slowly than normal prothrombin.

(6) PIVKAs differ from normal coagulation factors as to their adsorbability onto  $\text{Al}(\text{OH})_3$ .

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### INTRODUCTION

The 4 coagulation factors II (prothrombin), VII, IX and X have many properties in common. Their adsorption characteristics and molecular weights are very similar, therefore they are difficult to separate. Yet these factors can be separated and antibodies can be prepared that do not recognize common antigenic determinants<sup>1</sup>.

The available data upon primary structure and enzymatic activity suggest that they are strongly homologous proenzymes of serine esterases<sup>2-4</sup>. The activity of the 4 factors in the circulating plasma decreases in real vitamin K deficiency and in functional vitamin K deficiency induced by administration of vitamin K antagonists (coumarins or indandiones). From experiments on the kinetics of the blood coagulation process in normal and in vitamin K-deficient patients Hemker *et al.* inferred the existence of plasma proteins induced by vitamin K absence (PIVKAs) in the human<sup>5</sup>.

PIVKA may be defined as a protein occurring in plasma under conditions of vitamin K deficiency having antigenic determinants in common with one of the factors II, VII, IX and X, but possessing no activity in a coagulation test. As there are 4 vitamin K-dependent coagulation factors, one can postulate the existence of 4 PIVKAs. These proteins will be referred to as PIVKA-II, PIVKA-VII, PIVKA-IX and PIVKA-X, respectively.

PIVKA-II seems to be a well-defined entity, as it has been shown to occur in the plasma of humans and cows deficient in vitamin K or to whom vitamin K antagonists are administered. The protein from human origin converts into thrombin much more slowly than normal factor II<sup>6</sup> but like normal prothrombin obtains a thrombin-like activity when incubated with staphylocoagulase<sup>7,8</sup>. PIVKA-II precipitates antibodies against normal factor II. The protein shows the same electrophoretic mobility as normal factor II, but the mobility in the presence of calcium lactate is faster than that of normal prothrombin, and it is not adsorbed onto barium citrate in contrast to normal factor II<sup>9-12</sup>.

The observations that plasma of patients on anticoagulant treatment neutralize antibodies against the coagulation factors IX<sup>13</sup> and X<sup>14</sup> and contain a competitive inhibitor of factor X conversion<sup>15,16</sup> are indicative of the existence of PIVKA-IX and PIVKA-X in the human. In this article PIVKA-IX and PIVKA-X are demonstrated to occur in the anticoagulated cow besides PIVKA-II. It is also shown that these PIVKAs differ from the normal factors with respect to their adsorbability onto aluminum hydroxide. A preliminary report on the demonstration of these proteins appeared previously<sup>17</sup>.

#### MATERIALS AND METHODS

Normal bovine factors II, IX and X, used for the immunization of rabbits, were purified according to the method of Reekers<sup>18</sup>. Common inorganic chemicals, reagent grade, were obtained from Merck, Darmstadt, G.F.R. Marcoumar was a kind gift from Dr. R. M. Kunz (Hoffmann-La Roche, Basel, Switzerland). Agarose was a product of Koch-Light Laboratories Ltd. Aluminium hydroxide, moist gel, was purchased from British Drug Houses Ltd.

#### *Preparation of rabbit anti-bovine factor II, IX and X antiserum*

Antisera were prepared in rabbits by injection of purified preparations of the bovine factors II, IX and X. Antisera were treated with barium sulphate (100 mg/ml)

for 30 min at room temperature and heat inactivation was performed for 30 min at 57 °C. The adsorbed and heat inactivated antisera were centrifuged for 1 h at  $105,000 \times g$  (4 °C). Sodium mertiolate (0.01 % final concentration) was added before storage at -20 °C.

#### *Immunochemical methods*

Double immunodiffusion was performed according to Ouchterlony<sup>19</sup>. The one- and two-dimensional crossed immunoelectrophoresis was done according to Laurell<sup>20</sup>. Immunoprecipitation was performed after Kabat and Mayer<sup>21</sup>; mixtures of a fixed amount of antiserum (0.1 ml) plus a variable amount of antigens were incubated for 30 min at 37 °C and 72 h at 4 °C. The immunoprecipitate was collected by centrifugation for 1 h at  $5000 \times g$  followed by 4 washings with 0.15 M NaCl at 0 °C. The incubation mixtures contained 10 mM EDTA in order to prevent  $\text{Ca}^{2+}$ -dependent complement fixation to the immunoprecipitate<sup>22</sup>. After dissolution into 0.2 ml 0.2 M NaOH the protein content of the immunoprecipitate was determined according to Lowry *et al.*<sup>23</sup>.

Antiserum neutralization tests were carried out by incubation of 0.1 ml antiserum with the desired amount of plasma depending on the titer of antigen in plasma samples.

#### *Coagulation factor assays*

Coagulation times specifically dependent upon the concentrations of factor II, VII or X were obtained by mixing in a test tube in a water bath at 37 °C, 0.1 ml human plasma congenitally deficient in either factor II, VII or X, 0.1 ml human brain thromboplastin and 0.1 ml sample. The coagulation reaction was started by adding 0.1 ml of  $\text{CaCl}_2$  (33 mM). The moment of coagulation was assessed with the aid of a platinum hook. Coagulation times dependent upon the factor IX concentration were measured as described by Veltkamp *et al.*<sup>24</sup>.

Coagulation factor concentrations are expressed as a percentage of the content in normal bovine plasma. The concentrations were calculated from the coagulation times in specific tests as described by Hemker *et al.*<sup>25</sup> and Veltkamp *et al.*<sup>24</sup>.

Normal bovine plasma was obtained from a healthy cow, weighing about 550 kg, and 5 litres of blood were obtained by puncture of the jugular vein. The blood was centrifuged for 10 min at  $1000 \times g$  at 4 °C. The supernatant plasma was centrifuged for 30 min at  $12,000 \times g$ . The supernatant thrombocyte-poor plasma was stored in 1 ml and 5 ml vials at -20 °C.

Plasma under conditions of vitamin K absence was obtained from the same cow after phenprocoumon had been administered in two loading doses of 550 and 300 mg on two subsequent days and a daily administration of 250 mg/day for a further 21 days. The pertinent coagulation factor concentrations were: factor II, 8 %; factor VII, 12 %; factor IX, 10 %; factor X, 11 %. The other factors were in the normal range 80–100 %. This or similar plasmas will be referred to as coumarin plasma. Essentially the same results were obtained with coumarin plasmas from 5 other cows.



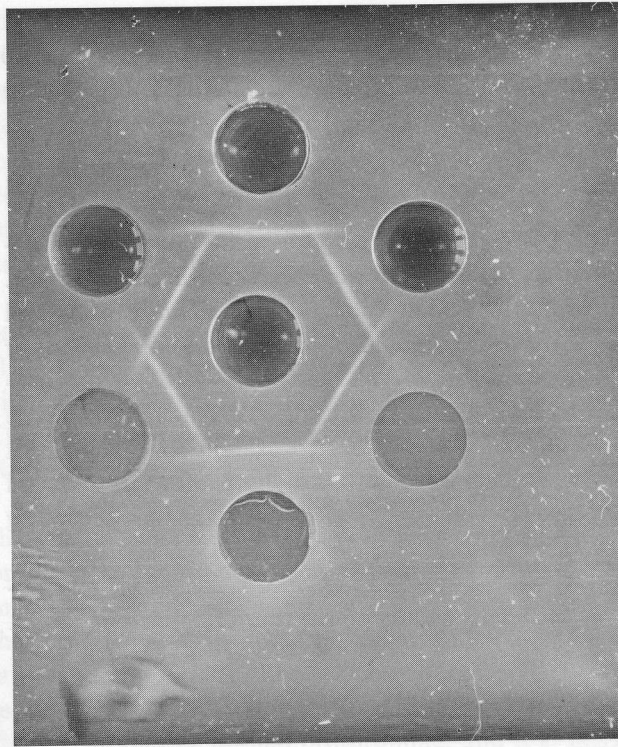


Fig. 1. Double immunodiffusion pattern of the antibodies used. Center well: normal bovine plasma; wells 1 and 4: anti-factor II antiserum; wells 2 and 5: anti-factor IX antiserum; wells 3 and 6: anti-factor X antiserum. Numbering starts at the top and is clockwise.

## RESULTS

### *Preparations of monospecific antisera against bovine factor II, IX and X*

The antisera developed against factor II, IX and X were monospecific. This is illustrated in Fig. 1 and Table I. The figure shows the non-identity of both antigens and antibodies. The table shows that the bovine coagulation factor activities II, IX and X are specifically inhibited by each of the three antibodies.

### *Qualitative analysis*

*Double diffusion immunoprecipitation experiments.* Double diffusion experiments for the detection of material precipitating with antibodies against factor IX are shown in Fig. 2. A substance can be demonstrated in normal plasma, coumarin plasma,  $\text{Al}(\text{OH})_3$ -adsorbed coumarin plasma,  $\text{Al}(\text{OH})_3$ -adsorbed coumarin serum and normal bovine serum that shows a reaction of complete identity with factor IX in normal plasma. The same can be demonstrated for factor X (Fig. 3). The results with antibody against factor II (not illustrated) were completely comparable to those shown in Fig. 2, as would be expected.

*Two-dimensional crossed electrophoresis.* The first of two-dimensional crossed

TABLE I

## NEUTRALIZATION OF COAGULATION FACTOR ACTIVITY BY ANTISERA

The antisera were treated with BaSO<sub>4</sub> and kept at 56 °C for 10 min which abolished all procoagulant activity. Antiserum (0.1 ml) was incubated for 1 h at 37 °C with 2.0 ml plasma diluted 1:5 with veronal acetate buffer, pH 7.35. Serum from non-immunized rabbits was used as a control. The coagulation times were obtained in a mixture of 0.1 ml sample, 0.1 ml tissue thromboplastin, and 0.1 ml human plasma congenitally deficient in the factors to be tested; for factor IX the method of Veltkamp<sup>24</sup> was used. The coagulation times are in seconds, means of 7 estimations.

Plasma	Antiserum against factor	Coagulation time obtained in a specifically deficient system			
		Factor II deficient	Factor VII deficient	Factor IX deficient	Factor X deficient
Bovine	II	49.3	23.8	138	34.1
Bovine	IX	27.3	23.4	450	31.4
Bovine	X	28.1	25.2	142	133.7
Bovine	control	27.6	23.8	135	34.4
Human	II	21.9	24.4	113	30.3
Human	IX	20.7	24.2	318	28.4
Human	X	20.5	22.7	111	27.9
Human	control	20.0	20.5	108	28.0
Buffer		133.0	126.3	365	135.5

electrophoresis experiments was carried out in the absence of calcium ions, or with the addition of calcium lactate (2.5 mM final concentration) to the sample, the buffer and the gel. When no calcium lactate was present, single peaks were obtained with both normal and coumarin plasma when run against either anti-factor IX or anti-factor X antibody (not illustrated). In the presence of calcium lactate the normal plasma still showed one peak, but the coumarin plasma showed a second peak (Figs. 4 and 5). From the plasma of a cow anticoagulated for 24 h two peaks of about equal height were obtained; one with an electrophoretic mobility equal to that in normal plasma, the other one faster. From the plasma of the same cow receiving phenprocoumon for 7 days, only the fast peak remained. It was also observed with factor II as has already been reported by others<sup>9,10</sup>.

#### Quantitative analysis

**Immunoprecipitation.** Immunoprecipitation curves of coumarin plasma with antibodies against factor II, factor IX, and factor X are shown in Figs. 6, 7 and 8. It can be seen that: (1) the amount of material precipitated is roughly equal in normal and in coumarin plasma although the coumarin plasma only has about 10 % of the coagulation factor activity of the normal plasma; and (2) the precipitation equivalence was not the same in normal and coumarin plasma. The latter finding shows that it will be impossible to obtain an exact quantitation of the PIVKAs by immunochemical means.

**One-dimensional crossed electrophoresis.** After homogenous distribution of antiserum in 1 % agarose, the amount of immunoreactive material present in plasma was determined by one-dimensional electrophoresis, according to Laurell<sup>20</sup>. The sur-

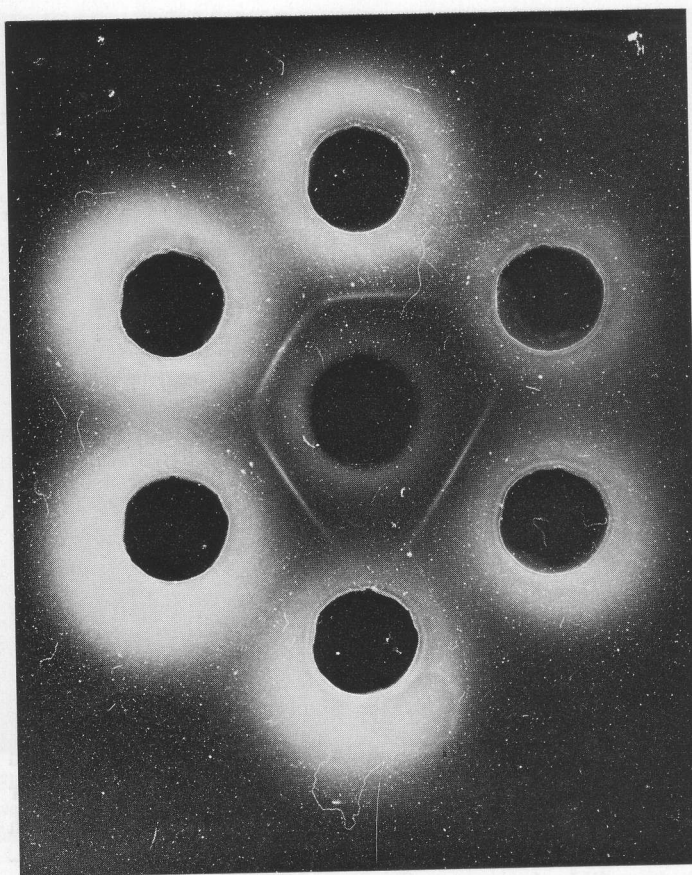


Fig. 2. Double diffusion immunoprecipitation with antibody against factor IX. Numbering starts at the top and is counterclockwise. 1: normal plasma; 2: coumarin plasma; 3:  $\text{Al}(\text{OH})_3$ -adsorbed coumarin plasma; 4:  $\text{Al}(\text{OH})_3$ -adsorbed normal plasma; 5:  $\text{Al}(\text{OH})_3$ -adsorbed coumarin serum; 6:  $\text{Al}(\text{OH})_3$ -adsorbed normal serum; center well: anti-bovine factor IX antiserum.

face under the immunoprecipitation peak or the peak length show a linear relation with the quantity of antigen applied. Fig. 9 shows the result of an experiment using anti-factor IX in the gel. Although the coumarin plasma used contained only 10% residual normal factor IX as determined by clotting factor assays, peak lengths were obtained slightly higher than those observed with normal bovine plasma. Aluminum hydroxide treatment of normal plasma or serum completely removed the antigen; aluminum hydroxide-adsorbed coumarin plasma still contained material precipitating with anti-factor IX. Identical results were obtained with anti-factor II and anti-factor X (Table II).

*Biological activity of PIVKA-II.* When coumarin plasma estimated to contain 20% normal factor II was incubated with an excess of prothrombinase (*i.e.* factor V and  $\text{X}_a$  and phospholipid; for details of the method see refs. 6 and 26), eventually an amount of thrombin is generated that is roughly equal to the amount obtained from a dilution of normal plasma containing 40% factor II. Adsorption with  $\text{Al}(\text{OH})_3$



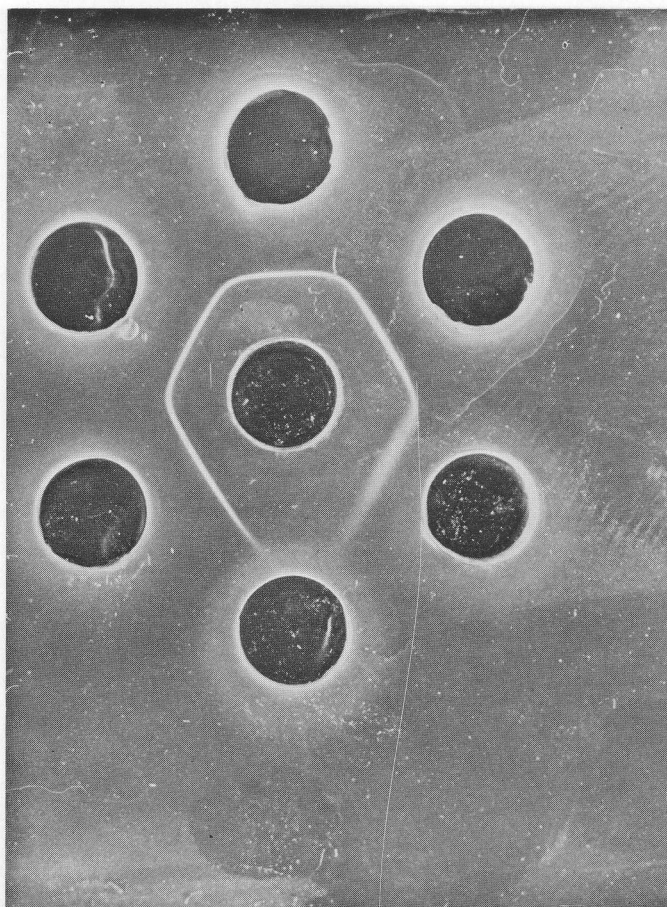


Fig. 3. Double diffusion immunoprecipitation with antibody against factor X. Numbering starts at the top and is counterclockwise. 1: normal plasma; 2: coumarin plasma; 3:  $\text{Al}(\text{OH})_3$ -adsorbed coumarin plasma; 4:  $\text{Al}(\text{OH})_3$ -adsorbed normal plasma; 5:  $\text{Al}(\text{OH})_3$ -adsorbed normal serum; 6:  $\text{Al}(\text{OH})_3$ -adsorbed coumarin serum; center well: anti-bovine factor X-antiserum.

caused normal plasma to lose all thrombin generating potency but roughly the half of the original amount could still be obtained from coumarin plasma after this treatment (Fig. 10).

#### DISCUSSION

A protein induced by vitamin K absence analogous to prothrombin (PIVKA-II as defined in the introduction) has been demonstrated by many investigators<sup>5,6,9,11,12,27</sup>. Our present results confirm these findings. The following arguments can be brought forward in support of the existence of PIVKA-IX and PIVKA-X besides PIVKA-II.

(a) Antibodies specific for one of the coagulation factors IX or X precipitate much more material from coumarin plasma than can be accounted for by the amount



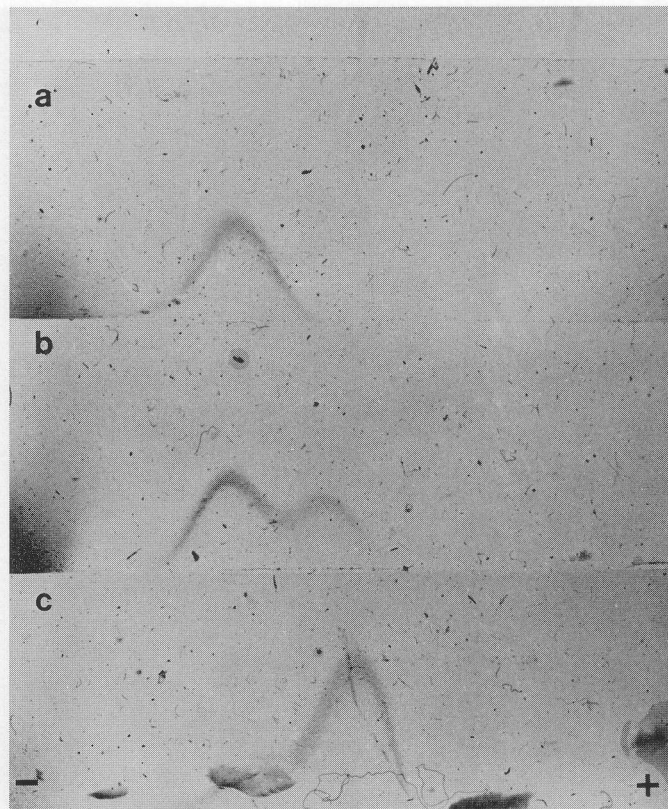


Fig. 4. Two-dimensional crossed electrophoresis of bovine plasmas in the presence of calcium lactate (first dimension, horizontal) and anti-factor IX antiserum (second dimension, vertical). a: normal plasma; b: plasma after 24 h anticoagulation; c: plasma after 100 h anticoagulation.

of normal factor IX or X in this plasma. Specific precipitation is improbable as a cause for this finding because the coagulation activity in the supernatant can be found only in the zone of antigen excess (unpublished observations).

(b) The materials precipitable with antibodies against factor IX found in normal plasma, coumarin plasma, normal serum, coumarin serum and  $\text{Al}(\text{OH})_3$ -adsorbed coumarin plasma show immunologically a reaction of complete identity. No precipitable material is found when normal plasma is adsorbed with  $\text{Al}(\text{OH})_3$  under circumstances known to remove all coagulation factor activity. A comparable observation is made with antibody against factor X.

(c) Quantitation of material reacting with the antibodies in a one-dimensional crossed immunoelectrophoresis according to Laurell<sup>20</sup> shows that more material is present in coumarin plasma and *a fortiori* in  $\text{Al}(\text{OH})_3$ -adsorbed coumarin plasma than can be accounted for by the activity level of the coagulation factors.

We conclude that PIVKA-IX and PIVKA-X exist besides PIVKA-II in the plasma of the cow receiving anticoagulants of the coumarin type. The PIVKA-II upon incubation with prothrombinase does produce thrombin, but at a much slower

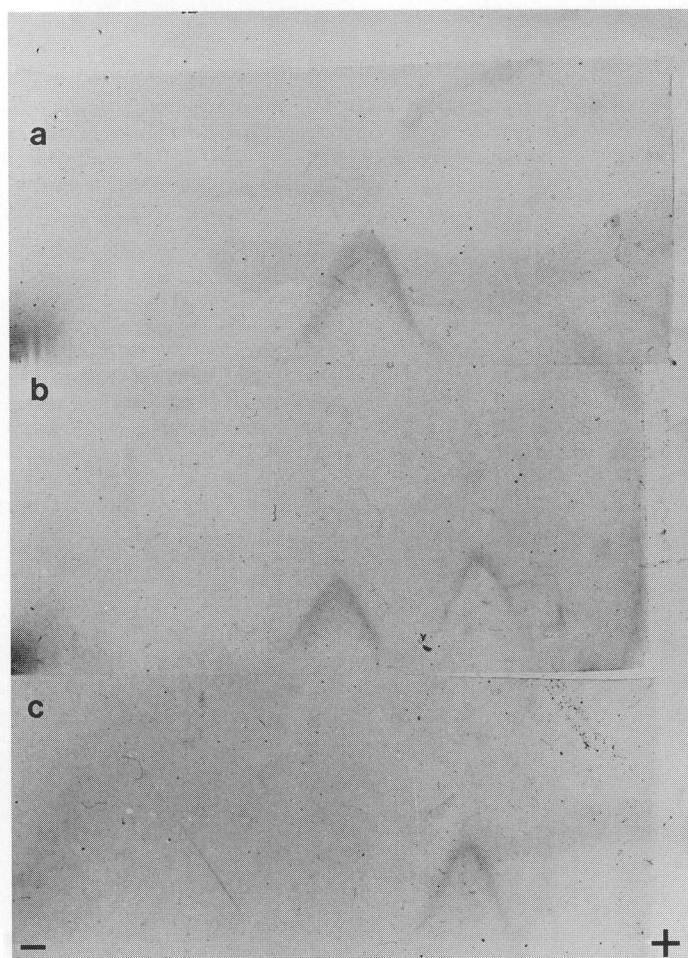


Fig. 5. Two-dimensional crossed electrophoresis of bovine plasmas in the presence of calcium lactate (first dimension, horizontal) and anti-factor X antiserum (second dimension, vertical). a: normal plasma; b: plasma after 24 h anticoagulation; c: plasma after 100 h anticoagulation.

rate than normal prothrombin. The reason for the occurrence of PIVKA-IX and PIVKA-X in the plasma of the anticoagulated cow has not yet been established. Our original hypothesis<sup>5</sup> to explain the occurrence of PIVKA has been that a block of the action of vitamin K causes an inhibition in a postribosomal stage of the prothrombin synthesis and that PIVKA is the intermediate piling up because of this block. The existing evidence as to prothrombin and PIVKA-I supports this view<sup>27-31</sup>. PIVKA-IX and PIVKA-X may arise in an analogous way.

Recent advances in chemical analysis of the difference between factor II and PIVKA-II suggest that the vitamin-K dependent mechanism serves to modify glutamic acid groups into  $\gamma$ -carboxyglutamic acid groups that strongly bind  $\text{Ca}^{2+}$  (ref. 32). The higher electrophoretic mobility in the presence of calcium ions suggests

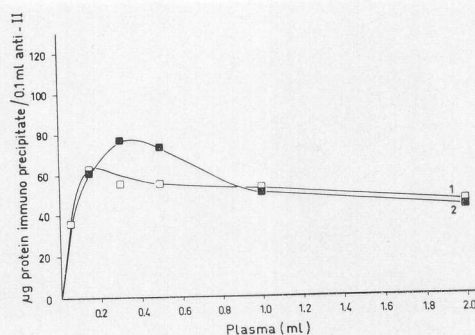


Fig. 6. Quantitative immunoprecipitation of factor II.  $\square$ , normal plasma;  $\blacksquare$ , coumarin plasma. The indicated amount of plasma was incubated with 0.1 ml of anti-factor II antiserum as described under Methods. In the zone of antibody excess, no factor II activity could be demonstrated in the supernatant; in the zone of antigen excess such activity was present.

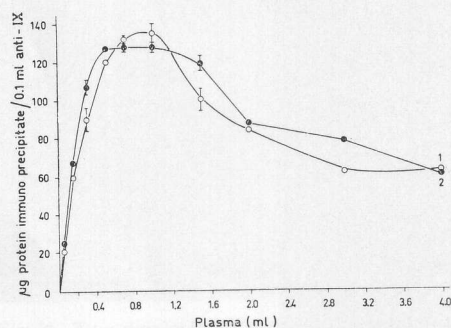


Fig. 7. Quantitative immunoprecipitation of factor IX.  $\circ$ , normal plasma;  $\bullet$ , coumarin plasma. The indicated amount of plasma was incubated with 0.1 ml of anti-factor IX antiserum as described under Methods. In the zone of antibody excess, no factor IX activity could be demonstrated in the supernatant; in the zone of antigen excess such activity was present.

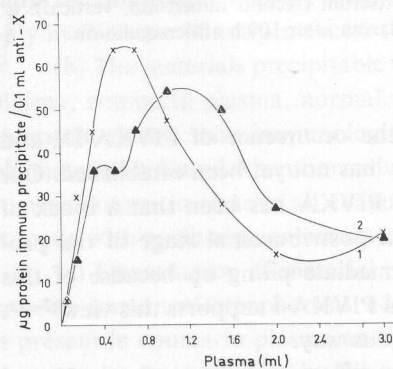


Fig. 8. Quantitative immunoprecipitation of factor X.  $x$ , normal plasma;  $\blacktriangle$ , coumarin plasma. The indicated amount of plasma was incubated with 0.1 ml of anti-factor IX antiserum as described under Methods. In the zone of antibody excess, no factor X activity could be demonstrated in the supernatant; in the zone of antigen excess such activity was present.



TABLE II

## QUANTIFICATION OF ANTIBODY PRECIPITABLE MATERIAL IN VARIOUS PLASMAS

The figures give the amounts assessed as a percentage of normal plasma. P, material demonstrable by one-dimensional crossed electrophoresis; B, biological activity. Adsorbed means that the sample is adsorbed by 1% (w/v) of  $\text{Al}(\text{OH})_3$ , adsorbed normal plasma showed neither precipitation nor biological activity. Means of 5 estimations.

	<i>Factor II</i>		<i>Factor IX</i>		<i>Factor X</i>	
	<i>P</i>	<i>B</i>	<i>P</i>	<i>B</i>	<i>P</i>	<i>B</i>
Normal serum adsorbed	37	15	60	66	73	80
adsorbed	0	0	0	0	0	0
Coumarin plasma	110	20	111	10	74	8
adsorbed	91	0	100	0	33	0
Coumarin serum	64	0	80	10	30	11
adsorbed	65	0	48	0	22	0

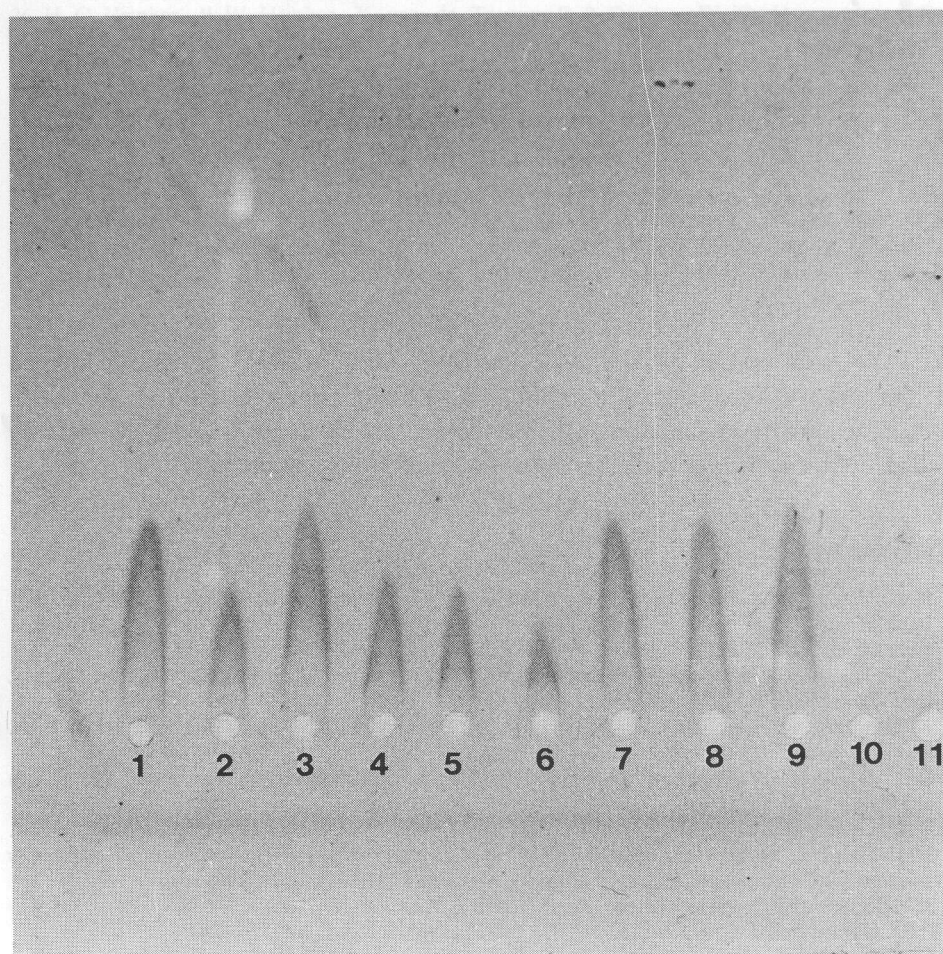


Fig. 9. One-dimensional crossed immunoelectrophoresis against anti-factor IX antibody. 1 and 2: normal plasma; 3 and 4: coumarin plasma; 5 and 6: normal serum; 7: coumarin serum; 8: ALCBP (adsorption time 5 min); 9: ALCBP (adsorption time 30 min); 10:  $\text{Al}(\text{OH})_3$ -adsorbed normal plasma; 11:  $\text{Al}(\text{OH})_3$ -adsorbed normal serum. Where two wells contained the same material, the second well contained this material in a 1:1 dilution.



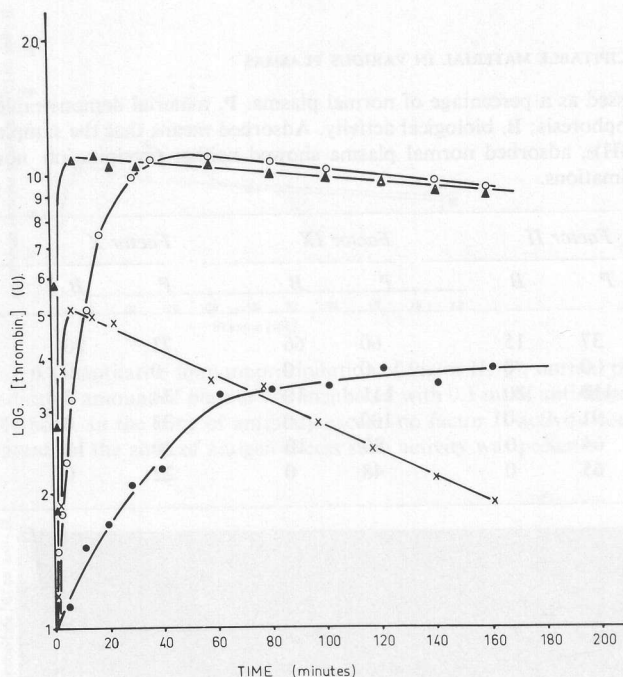


Fig. 10. Thrombin generation test. At zero time the prothrombin-converting mixture was added<sup>6</sup> and the amount of thrombin that developed is given as a function of time.  $\times-\times$ , normal plasma diluted 1:5;  $\blacktriangle-\blacktriangle$ , normal plasma diluted 1:2;  $\circ-\circ$ , dicoumarol plasma containing 20% prothrombin in the one-stage test;  $\bullet-\bullet$ , the same dicoumarol plasma adsorbed with  $\text{Al}(\text{OH})_3$  (1% w/v).

that PIVKA-IX and PIVKA-X, like PIVKA-II (ref. 12), lack the ability to bind calcium ions. As binding to a phospholipid-water interface via calcium ion is an essential feature in the function of the normal factors IX and X (ref. 33) the inability to bind  $\text{Ca}^{2+}$  may well account for the absence of biological activity.

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